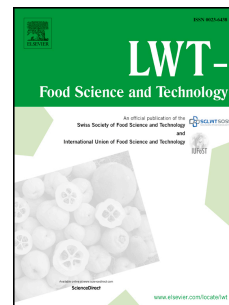


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**Proximal composition, sensorial properties and effect of ascorbic acid and  $\alpha$  - tocopherol on oxidative stability of bread made with whole flours and vegetable oils**

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*Abbreviations:* AACC, American Association of Cereal Chemists; ALA, alpha-linolenic acid; BHA, butylhydroxyanisole; CAA, Argentine Food Code; CO, canola oil; DSC, differential scanning calorimetry; FA, fatty acids; FF, flaxseed flour; HDL, high density lipoprotein; LDL, low density lipoprotein; L, extensibility; MDA, Malondialdehyde; MUFA, monounsaturated fatty acids; n3, fatty acids of the n3 family; n6, fatty acids of the n6 family; n9, fatty acids of the n9 family; n6/n3, relationship between fatty acids of series n6 and n3; OO, extra virgin olive oil; PV, Peroxide value; PUFA, polyunsaturated fatty acids; SF, whole soybean flour; SFA, saturated fatty acids; PUFA/SFA: relationship of polyunsaturated fatty acid and saturated fatty acids; P, tenacity; TBARS, thiobarbituric acid reactive substances; TCA, trichloroacetic acid; W, deformation energy; WB, wheat bran; WF, wheat flour.

## Abstract

Proximal composition, shelf-life, sensory properties and effects of ascorbic acid and  $\alpha$ -tocopherol on the oxidative stability of bread made with whole flours and vegetable oils were evaluated. Such effects were analyzed in two formulations: one with wheat flour + flaxseed flour + soybean flour + canola oil (F1), and the other with wheat flour + flaxseed flour + wheat bran + olive oil (F2). The proximal composition showed significant differences in moisture, fibre and carbohydrates due to the presence of wheat bran in one formulation. Omega 3 content in F1 was twice as high as that in F2, due to the contribution of flax meal and canola oil. Furthermore, both formulations presented good scores in the evaluated sensory attributes and a shelf-life of 2 days. The ascorbic acid in F1 produced a 40% reduction in primary lipid oxidation, while  $\alpha$ -tocopherol as antioxidant for F2 produced a 50% reduction in thiobarbituric acid-reactive substances and exerted a greater inhibiting effect than butylhydroxyanisole. Therefore, the fortification of wheat bread with whole flour and vegetable oils is an effective tool that allows to obtain functional food and the addition of antioxidants would be a good option to prolong the stability of multigrain bread studied.

## Keywords

Ascorbic acid,  $\alpha$ -tocopherol, oxidative stability, Omega-3 fatty acids, bread.

## 1 Introduction

There is a rising demand for the development of new foods with dietary properties (Dhen, Ben Rejeb, Boukhris, Damergi, & Gargouri, 2018). Bread can be fortified either by adding components that are removed in the milling process or by adding components that increase palatability or promote health (Hayta & Ozugur, 2011; Wandersleben et al., 2018). Moreover, the current trend in the industry is to introduce healthier fats into food products. Fats and oils, in general, can have a positive effect on food formulations for processing, quality, organoleptic and texture properties. But they can also have a negative effect when oxidized, particularly in the case of omega-3 (Hernandez, 2005). Omega-3 fatty acids can be effectively delivered through a variety of baked goods, such as several types of bread, nutritional bars, cereals and cookies (Hayta & Ozugur, 2011). Osuna, Judis, Romero, Avallone, & Bertola (2014) found that the addition of functional whole flours as flaxseed flour (FF), soybean flour (SF) and wheat bran (WB) produced an improvement in fatty acids compositions of traditional bread. As a result, FF and SF caused a decrease in saturated fatty acid (SFA) and monounsaturated fatty acid (MUFA) content, and an increase in polyunsaturated fatty acids (PUFA) in these types of bread. Thus, bread with FF increased considerably the content of omega-3. Similarly, bread substituted for mixtures of two flour samples (one with 16 g/kg FF+16 g/kg ST and the other with 16 g/kg FF+8 g/kg SF) showed the highest n3 content and good specific volume (Osuna, Romero, Judis, & Bertola, 2016). However, as mentioned above, the problem of foods enriched with omega-3 PUFA is that they are highly susceptible to lipid oxidation due to their high degree of unsaturation. Oxidation of unsaturated fatty acids produces a complex mixture of volatiles that significantly affects the sensory properties of foods, even when present in low quantities. The addition of antioxidants is, therefore, often necessary in order to

prevent oxidation (Genot, Kabri, & Meynier, 2013). The discovery of synthetic antioxidants has revolutionized the use of antioxidants in food. The effect of these antioxidants on bakery products was reviewed and found to be effective in enhancing shelf-life. Animal experimental studies have shown that some of the synthetic antioxidants had toxigenic, mutagenic, and carcinogenic effects. Hence, there is an increasing demand for the use of natural antioxidants in foods, especially in bakery products (Prabhasankar & Rao, 2010). A lipophilic antioxidant naturally present in wheat, particularly in WB, is  $\alpha$ -tocopherol. Moreover, ascorbic acid normally considered an antioxidant, also in bread, is used to achieve greater dough stability and bread volume (Grosch & Wieser, 1999), but the addition of antioxidants is not common for bread products. Enrichment of bread dough with antioxidants represents a strategy for producing loaves with a higher degree of oxidative stability and, hence, better overall sensory quality during storage. However, only few studies concerning the addition of antioxidants to bread products are available (Jensen, Ostdal, Skibsted, & Thybo, 2011).

In the present study the quality of bread made with mixtures of flour (wheat, flaxseed, wheat bran, and soybean) and oils (canola and olive) was evaluated during extended storage for oxidative and microbiological stability, and proximal and sensory analyses. Moreover, investigates the effect of adding naturally occurring antioxidants,  $\alpha$ -tocopherol and ascorbic acid, to multigrain bread.

## **2 Materials and methods**

### **2.1 Materials**

Commercial wheat flour (WF) (Florencia<sup>®</sup>, Argentina), used for all experiments, was provided by local stores, WF with the following parameters: deformation energy =  $334 \times 10^{-4}$  J, tenacity (P) = 135 mm, extensibility (L) = 61 mm, P/L = 2.21 (AACC

International, 2000), moisture =  $13.6 \pm 0.2\%$  and protein =  $11 \pm 0.3\%$  (Kjeldahl method, N x 5.7). SF (Ricedal Alimentos<sup>®</sup>, Argentina), FF (Vicentin<sup>®</sup>, Argentina), WB (Avecon<sup>®</sup>, Argentina), canola oil (CO) (Alimentos Fundación Favaloro<sup>®</sup>, Argentina), extra virgin olive oil (OO) (Mazza<sup>®</sup>, Argentina), and sodium chloride (Celusal<sup>®</sup>, Argentina) were used for the preparation of the multigrain bread. Compressed yeast (*Saccharomyces cerevisiae*, Calsa<sup>®</sup>, Argentina) was used as a leavening agent. The moisture content of yeast was 71.5%.

The added antioxidants were:  $\alpha$ -tocopherol (Tanvimil<sup>®</sup>E), ascorbic acid, and butylhydroxyanisole (Sigma<sup>®</sup>).

## 2.2 Bread-making process

### 2.2.1 Ingredients for the formulations

Both formulations made with mixtures of flour and oils were obtained and selected in previous studies (Osuna, Romero, Avallone, Judis, & Bertola, 2018).

*Base formulation 1 (F1):* Total Flour (100 g with 97.6 g wheat flour, 1.6 g flaxseed flour, and 0.8 g soybean flour), commercial compressed yeast (4% flour basis), canola oil (4% flour basis), sodium chloride (2% flour basis), and tap water up to optimum absorption (55% flour basis).

*Base formulation 2 (F2):* Total Flour (100 g with 96.8 g WF, 1.6 g FF, and 1.6 g WB), commercial compressed yeast (4% flour basis), OO (4% flour basis), sodium chloride (2% flour basis), and tap water up to optimum absorption (55% flour basis).

### 2.2.2 Preparation of bread and storage conditions

Multigrain bread was prepared using the straight dough method (Osuna et al., 2016). All the ingredients were weighed and mixed at high speed with a rapid mixer (Zonda<sup>®</sup>, Buenos Aires, Argentina) for 7 min. After a resting time of 15 min at room temperature, the dough was laminated into the laminator (Rd<sup>®</sup>, Buenos Aires,

Argentina) and allowed to stand for a 15 min period. Then the dough was divided into loaves of 200 g and modelled manually. The loaves were formed and placed into aluminium pans (24.5 × 6.5 cm). These were placed in a fermentation chamber (90 min, 35 °C, 85 RH%) and baked in an electric oven (Zonda®, Buenos Aires, Argentina) for 15 min at 180 °C. Multigrain bread was cooled for 2 h. Then, some loaves were subject to physicochemical analysis and others were packed in polyethylene bags and stored at 25 °C ± 1 °C for 3, 6 and 10 days. The experiment was replicated at least twice.

### 2.3 Shelf-life of bread

In order to study multigrain bread quality during storage, two samples were prepared for each base formulation: one with the preservative sodium propionate (0.38% flour basis), and the other without preservative. For this, moisture content, water activity, the retrogradation of starch, crumb hardness and the microbiological analyses were measured. The analyses were performed at 0, 3, 6 and 10 days of storage.

#### 2.3.1 Moisture content

Moisture content of the crumbs and crusts was determined at each sampling time in accordance with AOAC (2005).

#### 2.3.2 Retrogradation of starch

A calorimetric analysis was carried out by means of a DSC 823 (Mettler-Toledo, Zurich, Switzerland). A sample of approximately 10 mg was taken from multigrain bread and tightly packed into an aluminium pan. The pan was closed with a lid and weighed. All the samples were heated from 25 to 120 °C, at 10 C/min. Melting heat of retrograded starch ( $\Delta H$ ), initial transition temperature ( $T_o$ ), peak transition temperature ( $T_{pk}$ ) and final transition temperature ( $T_f$ ) were calculated. Average values of three measurements were calculated for each sample.

### 2.3.3 Microbiological analyses

Ten grams of each sample were aseptically taken and transferred into a stomacher bag with 90 ml of sterile peptone water in order to be homogenized. Appropriate dilutions of the homogenates were inoculated into several growth media to determine viable counts: yeast and mold in Chloramphenicol Sabouraud agar (25 °C, 5 days) and total aerobic mesophilic bacteria on plate count agar (Karaoglu, Kotancilar, & Gurses, 2005) after incubation at 30 °C for 72 h.

### 2.3.4 Crumb hardness

Hardness measurements were evaluated by Texture profile analysis (TPA) by using a Textural Analyzer (CT3, Brookfield, USA). Multigrain bread (6 parallel samples) were sliced into 20-mm thick slices and the center of the bread was cut with a round mold (20 mm of diameter and 20 mm height). The multigrain bread cylinders were compressed in two successive cycles using TA 25/1000 acrylic cylinder probe (50.8 mm diameter), 50% penetration depth and a test speed of 0.5 mm/s. Hardness values were expressed as N (Osuna et al., 2014).

## 2.4 Fatty acids and proximal composition, calorific value and sensory analysis

### 2.4.1 Fatty Acid Composition

Total lipids were extracted using the Bligh & Dyer method (1959). Fatty acid (FA) composition was determined by gas chromatography (GC), after derivatization of the extracted oils to fatty acid methyl esters (FAME) in accordance with the AOAC Official Method 969.33 (2005), using an Agilent gas chromatograph (model 6850A HP, Agilent Technologies Inc, CA, USA), equipped with a 60 m Supelco 2340 capillary column. The temperature of the injector and the detector was kept at 250 °C. The injected volume was 1.0 µl. The carrier gas was helium at 0.6 µl min<sup>-1</sup>. The Split ratio used was 1:100. The temperature of the column was kept at 140 °C for 5 min, raised to 240 °C at



4 °C/min, and maintained at 240 °C for 10 minutes. Fatty acids (FA) were identified by comparing their retention times with international standards (Supelco 37 Components FAME Mixture, Bellefonte, PA), and reported as g 100g<sup>-1</sup> of total FA. Results were expressed as relative quantities of SFA, MUFA, and PUFA; PUFA/SFA ratio; and n6/n3 ratio.

#### 2.4.2 Proximal composition

Moisture, ash and protein content (micro-Kjeldahl method), lipid content (Soxhlet method 945.16), and crude fibre were analyzed in the final product following the methods of AOAC (2005). Carbohydrate content was determined by Antrona method (Southgate, 1976). The caloric value was calculated using the Atwater conversion factors: 9 kJ/g of lipid, 4 kJ/g of carbohydrate, and 4 kJ/g of protein (Zou, Moughan, Awati, & Livesey, 2007).

#### 2.4.3 Sensory analysis

A sensory panel of 90 untrained panelists (55 women, 35 men) from 18 to 60 years old, who like bread or are regular consumers of bread, was set up to evaluate the acceptance of the substitute bread. Samples were randomly coded and presented to the panel members in trays with instructions for the evaluation. The analyzed attributes were appearance, color, texture, taste and overall acceptability, using a nine-point hedonic scale for each parameter. The panelists were asked to indicate the score of each sensory descriptor and a score for overall acceptability on a scale ranging from 1 (extremely dislike) to 9 (extremely like). A five score was used as a minimum threshold for acceptability.

#### 2.5 Oxidative stability

Lipid oxidation of multigrain bread samples during storage was monitored by measuring the formation peroxide value and thiobarbituric acid-reactive substances. The analyses were performed at 0 and 6 days of storage.

#### 2.5.1 Antioxidants addition

In order to increase oxidative stability, samples with natural antioxidants ( $\alpha$  – tocopherol and ascorbic acid) and with synthetic antioxidant (BHA) were made for each base formulation. The ingredients of the formulations for oxidative stability are shown in Table 1.

#### 2.5.2 Oil extraction for oxidative stability assay

Lipids were extracted from multigrain bread using the Bligh & Dyer (1959) method. Samples of 30 g were milled with 28.3 ml ultrapure water, 21.7 ml chloroform, and 51 ml methanol. After homogenization, 32 ml of ultrapure water and 23.5 ml of chloroform were added. The mixture was transferred to a separate funnel. Bottom phase containing oil in chloroform was separated and desorbed by solvent evaporation at room temperature under nitrogen flow until constant weight. Extracted oil was stored in sealed vials at -18 °C under nitrogen atmosphere.

#### 2.5.3 Peroxide value

Peroxide value (PV) was determined in accordance with the official method developed by the International Dairy Federation, with minor modifications described by Vasile, Romero, Judis, & Mazzobre (2016). First, 0.008 g of extracted oil was dissolved in 9.9 ml of a mixture of chloroform–methanol (70:30), and 0.05 ml of ammonium thiocyanate (0.3 g/ml) were added and spectrophotometrically measured by absorbance at 501 nm to obtain  $E_0$ . Then, 0.05 ml of iron (II) chloride solution was added and, after 5 min of reaction time, absorbance of the red iron (III) complex was determined ( $E_2$ ).

191 The results were expressed as milliequivalents of oxygen per kilogram of extracted oil  
 192 (meq O<sub>2</sub>/kg fat), defined by:

$$PV = \frac{[E_2 - (E_0 + E_1)]}{55.84 \times m_0}$$

193 where E<sub>1</sub> is the absorbance at 501 nm of reagent blank, and m<sub>0</sub> is the mass (g) of the  
 194 tested oil portion.

#### 195 2.5.4 Thiobarbituric acid-reactive substances

196 A modified thiobarbituric acid-reactive substances (TBARS) method in accordance with  
 197 AOAC Official Method Cd 19–90 was followed to evaluate the extent of lipid oxidation  
 198 (Romero et al., 2014), using an UV-Vis Evolution 600 Thermo Scientific®  
 199 spectrophotometer. One hundred milligrams of extracted lipids were taken, and the  
 200 following reagents were sequentially added: 100 µl (BHA 36 g/l) and 2 ml of  
 201 thiobarbituric acid / trichloroacetic acid (TBA/TCA) solution (20 mM TBA in 150 g/l  
 202 TCA). The mixture was heated in a 90 °C water bath for 15 min and cooled at room  
 203 temperature. Afterwards, two milliliters of chloroform were added and the mixture was  
 204 centrifuged at 1.000 rpm for 15 min. The absorbance of the supernatant was measured at  
 205 532 nm in a spectrophotometer against a blank containing 0.1 ml H<sub>2</sub>O and 2 ml  
 206 TBA/TCA solution. Malondialdehyde (MDA) standard curves were prepared by  
 207 1,1,3,3-tetramethoxypropane and TBARS were expressed as mg/kg of MDA  
 208 equivalents of samples.

#### 209 2.6 Statistical analysis

210 INFOSTAT statistical software (Facultad de Ciencias Agropecuarias, UNC, Argentina)  
 211 was used to perform the statistical analysis. Determinations were done at least three  
 212 times. A Tukey's test was done in order to evaluate differences among samples  
 213 (significant level at p < 0.05).

### 3 Results and discussion

#### 3.1 Shelf-life of bread

Table 2 shows the effect of storage time on moisture content, water activity and crumb hardness in both types of multigrain bread. Moisture content tended to decrease in all the analyzed bread as the days of storage passed. The moisture content of bread made with CO and preservative (F1 + P) showed significant differences ( $p < 0.05$ ) with the rest of the formulations at day 0. After baking, there was a moisture gradient between crust and crumb that tended to equilibrate during storage. In a closed system, water would equilibrate between the crumb and crust during storage (Baik & Chinachoti, 2000). The results obtained here were lower than those obtained by Baik & Chinachoti (2000) for white bread with preservative (32%), but the total decrease in moisture was higher in white bread than in the multigrain bread analyzed in this study. Similarly, Ammar et al. (2016) found a lower decrease of moisture in the loaves with alhydwan flour than in the white bread. Fik, Surówka, Maciejaszek, Macura, Michalczyk (2012) found that the moisture content of the calcium-fortified wholemeal crumb was gradually reduced during storage.

The water activity of the analyzed multigrain bread ranged between 0.93 and 0.96 and decreased as the days passed ( $p < 0.05$ ), with the lowest water activity for F1+P at day 10. Kotancilar, Gerçekaslan, and Karaoğlu (2009) studied the effects of bread weight and storage time on the values of water activity of traditional Vakfikebir bread. They reported that the water activity of the crumb decreased slightly while the water activity of the crust increased greatly ( $p < 0.01$ ) during storage in the analyzed bread with different weight. Texture is an essential quality attribute for bakery products. Crumb hardness is commonly used as an indicator of bread staling, and it is negatively correlated with bread quality (Liu, Meng, Shan, Jin, & Wang, 2010). After only 3 days

of storage, negative changes in the texture of crumb were recorded with an increase in hardness from 98 to 200% in the bread with the same formulation. Between the different formulations, the hardness of multigrain bread had significant differences ( $p < 0.05$ ) at day 0, presenting F2 the greater hardness. This is due to the presence of WB in the formulation. This is in accordance with Fik et al (2012), who observed negative changes in hardness parameters in calcium-enriched wholemeal loaves as the storage days passed. Besbes, Jury, Monteau and Le Bail (2014) concluded that bread crumb stiffness increases as moisture level decreases, especially in the part of the bread that is subject to a higher heating rate (crust). Therefore, the increase in firmness is strongly related to the transfer of water on a macromolecular scale between the crumb and the crust.

*Thermal properties.* In fresh bread, the crust is dry, hard and brittle—because both the starch and the protein are in the vitreous state. During bread staling, water migrates from the crumb to the crust, and the proteins and starch go from the vitreous state to the gummy state. At the same time, there is a hardening in the crumb. In bread staling, the reorganization of both the amylose and the amylopectin takes place, with an increase in the crystallinity and, therefore, the rigidity of the network (Hug-Iten, Escher, and Conde-Petit, 2003).

When a sample of aged bread is heated in the DSC capsule, a new endotherm is observed and its temperature peak is related to the temperature at which the amylopectin melts. The enthalpy change associated with that phase transition can be measured. Because the time scale in which the endotherm develops and the increase in the firmness of the crumb are globally similar in magnitude, the DSC can be used to quantify the rate of bread staling (Jagannath, Jayaraman, and Arya, 1999).

Table 3 shows the thermal properties of bread made with flour mixture and oils during storage. Both formulations had similar behaviour during storage since no significant differences were observed in the retrogradation parameters ( $T_0$ ,  $T_f$ ,  $\Delta T_r$ , and  $\Delta H$ ). The added ingredients produced virtually no alteration in the transition temperature range. In addition, the entropy of retrogradation tended to be lower in samples with the WF+FF+SF+CO mixture, but the decrease was not significant ( $p>0,05$ ). The evolution of amylopectin retrogradation (one of the main mechanisms involved in bread ageing) was lower in the formulation with SF and CO. Addition of SF showed to decrease amylopectin recrystallization and the percentage of increase in firmness during storage (Vittadini and Vodovotz, 2003).

*Microbiological analyses.* The results obtained by the variation of the content of total mesophilic aerobes of each of the multigrain bread formulated with and without preservative (calcium propionate) are shown in Figure 1. Microbiological analysis of the bread prior to storage showed that the initial total bacteria count as well as yeast and mold levels were below 10 CFU/g. The count of mesophilic aerobic bacteria in the bread showed that the growth of microorganisms was practically insignificant in the first 3 days for all the formulations. The count of microorganisms for the bread without preservatives was suspended because, at day 6 of storage, mold was detected at first glance on the surface of the bread. Regarding mold and yeast counts, formulations without antimicrobials showed higher growth compared to those added with antimicrobials.

According to the Latin American norm (Official Mexican Standard NOM-147-SSA1-1996, 1996), the maximum limit of aerobic mesophiles allowed in bread is  $1 \times 10^3$  CFU/g and of mold and yeast, 20 CFU/g. This indicates that the bread obtained in this study is within the established limits of aerobic mesophiles. However, the count of mold and

yeast only remained below these limits with the addition of antimicrobials. It was possible to establish that the multigrain loaves made with antimicrobials were the most stable microbiologically, with a maximum time of stability of 7 days.

### **3.2 Fatty acids and proximal composition and fatty acids and sensory analysis**

Table 4 shows the results of chemical composition, lipid profile, caloric value and hedonic tests of both wheat flour bread supplemented with different mixtures of flour and oils. The results showed that both mixtures reflect significant differences ( $p < 0.05$ ) in the moisture content, carbohydrates and crude fibre of these products. This could be due to the WB content of one of the formulations since both have equal content of FF. The bran increased the carbohydrates and raw fibre content and produced high water retention. In a recent publication, Jacobs, Hemdane, Dornez, Delcour, & Courtin, (2015) reported that water-binding mechanisms on macro-, micro-, and nanoscale, and on a molecular level allow bran to retain water either weakly or strongly. Moreover, bran is rich in polysaccharides, which can bind water on a molecular level through the formation of hydrogen bridges (Chaplin, 2003).

Regarding fatty acid composition, it was found that omega 3 contents, i.e. (ALA), were of 22.69 and 11.22 (g/100 g of total fatty acids) for F1 and F2, respectively. Therefore, F1 presented twice as many n3 fatty acids as F2, due to the contribution of both flax meal and canola oil. On the other hand, the content of oleic acid was higher in F2, due to the contribution of olive oil. Simopoulos (2008) studied the importance of n6/n3 ratio in Western diets and concluded that low ratios (1 to 2) are associated with a reduced risk of several chronic diseases, such as cardiovascular diseases, inflammatory diseases, and cancer. Therefore, the n6/n3 relationship can be used to assess the nutritional quality of the lipid fraction of foods. The n6/n3 ratios calculated in this study for the F1 and F2 were 1.09 and 2, respectively. Thus, these results are consistent with

the values previously reported by Simopoulos (2008), and hence, the consumption of this fortified bread could contribute to restoring the FA balance of the diet. Moreover, to indicate whether a particular food is healthy, the PUFA/SFA ratio should be greater than 0.45, according to the World Health Organization (WHO, 2008). The PUFA/SFA ratios of both bread formulations were within these recommended values, but F2 presented a lower ratio due to the contribution of the palmitic acid from the olive oil and the wheat bran, among the SFA (Osuna et al., 2014).

Figure 3 shown appearance visual of the slice of bread prepared from flours mixture and oils, F1 and F2. Few differences were observed, F1 being darker the crumb and crust than F2. Thus, the sensory analysis showed that both multigrain bread made with WF+FF+SF+CO or WF+FF+WB+OO presented good scores in the evaluated attributes and that there were no significant differences ( $p>0.05$ ) in taste, texture and general acceptability. However, in color, the F1 received a significantly higher score than bread with F2. This difference was due to the addition of different flour blends to the bread formulations, affecting the sensory property of color. Sęczyk, Świeca, Dziki, Anders, & Gawlik-Dziki (2017) found that the incorporation of up to 4% flaxseed hulls had a slightly unfavourable effect on the sensory properties of bread but ensured satisfactory consumer acceptability. Marpalle, Sonawane, & Arya (2014) showed that enrichment of bread with 15% of ground flaxseeds negatively affected the taste and overall acceptability – received scores were below the level of acceptance. The results of this study presented good scores for overall acceptance coinciding with bread with equal substitution levels.

### **3.3 Oxidative stability**

The primary and secondary products formation of the lipid oxidation of multigrain bread after cooking and at the end of storage showed different behaviours for each base



formulation. The peroxide values for F1 were greater than 4 milliequivalents of oxygen per kilogram of extracted oil, showing no significant differences ( $p > 0.05$ ) at day 0 and at day 6 of storage, while that the production of peroxide for F2 after cooking was minor and significantly different ( $p < 0.05$ ) that the value found at the end of the storage (1.88 and 3.62 meq  $O_2$ / kg extracted oil respectively). The higher content of polyunsaturated fatty acids from canola oil could explain the higher rate of peroxides production in the F1 (WF+FF+SF+CO). Respect to TBARS values, it was observed significant differences ( $p < 0.05$ ) at day 0 and at day 6 for both formulations, although its evolutions had opposite behaviour. The TBA reactive substances formation for F1 decreased at the end of storage at 25 °C after reaching values of 6.97 to 4.22 mg MDA/kg sample; while for F2, the concentration of the secondary compounds of the oxidation was increased of 6.79 mg MDA/ kg (at 0 days) to 11.75 mg MAD/ kg sample (at 6 days). Reactions similar to Maillard's between amino acids from soya protein and aldehydes and ketones from lipid oxidation in the F1 would cause the decrease of the TBA reactive substances. This behaviour was reported by several authors (Zhang, Xiao, & Ahn, 2013)(Hidalgo & Zamora, 2000).

Marpalle, Sonawane, LeBlanc, and Arya (2014) studied the oxidative stability of bread made with 10 g/100 g roasted ground flaxseed and observed an increase in peroxide value of 6.6 to 13 meq  $O_2$ /kg sample with days of storage. The results of this investigation were lower than those reported by Marpalle et al. (2014), probably because the formulations were made with minor amount of flour and oils as PUFA sources.

As the products of lipid oxidation influence food quality and are considered potentially toxic for the human health, antioxidants are used for decrease the oxidative deterioration.

The peroxide and TBA-reactive substances of the bread made of flour mixtures and oil, and with and without the antioxidant additives (ascorbic acid,  $\alpha$ -tocopherol and BHA) after cooking and at the end of storage (6 days) can be observed in Figure 2.

The addition of antioxidants in multigrain bread made with WF+FF+SF+CO (F1) caused a significant decrease ( $p < 0.05$ ) in PV at day 6 of the storage (Figure 2). It should be noted that when the PV was analyzed, ascorbic acid was the most effective additive presenting a 40% reduction with respect to the formulation without antioxidants (F1); though, it did not reach the reduction percentage of the synthetic antioxidant BHA (71%). On the other hand, the TBARS values were not significantly different ( $p > 0.05$ ) and presented a slight reduction at the end of the storage, likely due to the reaction of secondary oxidation products with the amino acids of the soybean flour proteins in an oxidizing system as behaviour of your base formulation.

In the multigrain bread made with WF+FF+WB+OO (F2), the two natural antioxidants tested caused oxidation inhibition in both indicators, and, in this case, the TBA acid-reactive substances were the most affected parameter. The reduction in the formation of the hydroperoxides reached a 22% for the sample with  $\alpha$ -tocopherol ( $p > 0.05$ ), comparable to the formulation with synthetic antioxidant BHA (28%), and caused a 50% reduction ( $p < 0.05$ ) in the TBA-reactive substances exerting a stronger inhibitory effect than BHA.

Therefore, ascorbic acid is the antioxidant of choice to preserve oxidative stability of bread formulations made with CO and FF during storage. Cachaper (2005) studied the effect of different natural and synthetic antioxidants on the inhibition of the oxidation of loaves with 15% of flax and ascorbic acid, which was not as effective as BHA or the combination of BHA-BHT (butylhydroxytoluene) in the prevention of lipid oxidation. Frankel (1996) reviewed the effect of antioxidants in inhibiting hydroperoxide

formation and may thus be critical in preserving food quality by reducing rancidity due to aldehyde formation. Natural antioxidants like tocopherols and ascorbic acid can interrupt lipid autoxidation by interfering either the chain propagation or the decomposition process.

#### 4 Conclusion

The proximal composition of both formulations showed differences in moisture, fibre and carbohydrate content, due to the presence of WB in one formulation. The omega-3 content in WF+FF+SF+CO was twice as high as that in WF+FF+WB+OO, due to the contribution of flaxseed and canola oil. Given these facts, the addition of the mixtures of flour (wheat, flaxseed, wheat bran, and soybean) and oils (canola and olive) could be beneficial food additives for obtaining functional bread. Both formulations showed good sensory scores, presenting only significant differences in the color attribute. Regarding the shelf-life, it was 2 days for both formulations. The study of the effect of adding naturally occurring antioxidants,  $\alpha$ -tocopherol and ascorbic acid, to bread made with mixtures of flour (wheat, flaxseed, wheat bran, and soybean) and oils (canola and olive) during extended storage indicated that both had antioxidant effects. Ascorbic acid in the WF+FF+SF+CO formulation reduced 40% of peroxide value, and  $\alpha$ -tocopherol as an antioxidant for the WF+FF+WB+OO combination caused a 50% reduction in the thiobarbituric acid-reactive substances. Therefore, the addition of ascorbic acid and  $\alpha$ -tocopherol would be a good option to prolong the stability of PUFA of the bread made with mixtures of whole flour and vegetable oils.

To sum up, the fortification of wheat bread with whole flour and vegetable oils (WF+FF+SF+CO or WF+FF+WB+OO) is an effective tool that allows to obtain functional food with a significantly enhanced nutraceutical potential and the addition of

natural antioxidants would be a good option to prolong the stability of polyunsaturated fatty acids of the breads studied.

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542

## Figure Captions

**Figure 1.** Variation of microbial content, (A) total mesophilic aerobic bacteria and (B) molds and yeasts, in the bread made with flours mixture and oils, F1 made with wheat flour + flaxseed flour +soybean flour + canola oil (black circle), F1+P made with wheat flour + flaxseed flour +soybean flour + canola oil + preservative sodium propionate (grey circle), F2 with wheat flour + flaxseed flour +wheat bran + olive oil (black triangle) and F2+P with wheat flour + flaxseed flour +wheat bran + olive oil + preservative sodium propionate (grey triangle) during storage days.

**Figure 2.** The peroxide value and TBA reactive substances of the breads made with flours mixtures and vegetable oils and with and without the antioxidant additives (Ascorbic acid,  $\alpha$ -tocopherol and butylhydroxyanisole - BHA) after cooking and at the end of storage (6 days). <sup>a. b. c. d</sup> indicates significant differences between the different formulations on the same day of storage. *Abbreviations:* F1 and F2, base formulations without antioxidants; F1-C, formulation 1 with ascorbic acid; F1-E, formulation 1 with  $\alpha$ -tocopherol; F1-BHA, formulation 1 with BHA; F2-C, formulation 2 with ascorbic acid; F2-E, formulation 2 with  $\alpha$ -tocopherol and F2-BHA, formulation 2 with BHA.

**Figure 3.** Appearance visual of the slice of bread prepared from flours mixture and oils, F1 made with wheat flour + flaxseed flour +soybean flour + canola oil (left) and F2 with wheat flour + flaxseed flour +wheat bran + olive oil (right)

Table 1. Formulations of breads with addition of antioxidants

Ingredients (g)	Samples							
	F1	F1-C	F1-E	F1-BHA	F2	F2-C	F2-E	F2-BHA
WF	976	976	976	976	968	968	968	968
FF	16	16	16	16	16	16	16	16
SF	8	8	8	8	-	-	-	-
WB	-	-	-	-	16	16	16	16
CO	40	40	40	40	-	-	-	-
OO	-	-	-	-	40	40	40	40
Ascorbic acid (mg)	-	28.9	-	-	-	29.5	-	-
$\alpha$ -tocopherol (mg)	-	-	28.9	-	-	-	29.5	-
BHA (mg)	-	-	-	11.56	-	-	-	11.8
Preservative	3.8	3.8	3.8	3.8	3.8	3.8	3.8	3.8
compressed yeast	40	40	40	40	40	40	40	40
Salt	20	20	20	20	20	20	20	20
Water	540	540	540	540	540	540	540	540

*Abbreviations:* WF, wheat flour; FF, flaxseed flour; SF, soybean flour; WB, wheat bran; OO, olive oil; CO, canola oil; : F1 and F2, base formulation without antioxidants; F1-C, formulation with ascorbic acid; F1-E, formulation with  $\alpha$ -tocopherol; F1-BHA, formulation with BHA; F2-C, formulation with ascorbic acid; F2-E, formulation with  $\alpha$ -tocopherol and F2-BHA, formulation with BHA.

**Table 2. Effect of storage time on moisture content, water activity and hardness of crumb in both wholemeal bread.**

Days	Moisture content (%)				Water activity				Hardness			
	F1	F1+P	F2	F2+P	F1	F1+P	F2	F2+P	F1	F1+P	F2	F2+P
0	33.34±0.26 <sup>bb</sup>	31.8±0.15 <sup>aa</sup>	32.9±0.26 <sup>bb</sup>	33.1±0.92 <sup>bb</sup>	0.96±0.01 <sup>ab</sup>	0.96±0.01 <sup>ad</sup>	0.96±0.02 <sup>ab</sup>	0.96±0.01 <sup>ad</sup>	1.35±0.03 <sup>abA</sup>	1.22±0.07 <sup>aA</sup>	1.69±0.06 <sup>ca</sup>	1.52±0.14 <sup>bca</sup>
3	32.30±0.44 <sup>aA</sup>	31.86±0.19 <sup>aA</sup>	32.11±0.16 <sup>aA</sup>	32.2±0.67 <sup>aA</sup>	0.96±0.02 <sup>bcAB</sup>	0.95±0.01 <sup>abC</sup>	0.96±0.01 <sup>cB</sup>	0.95±0.01 <sup>aC</sup>	2.68±0.64 <sup>aB</sup>	3.68±0.75 <sup>aB</sup>	2.95±0.31 <sup>aB</sup>	3.68±0.26 <sup>aB</sup>
6	31.80±0.31 <sup>ba</sup>	31.28±0.68 <sup>aa</sup>	31.6±0.15 <sup>abA</sup>	31.3±0.05 <sup>abA</sup>	0.95±0.02 <sup>ba</sup>	0.94±0.02 <sup>aB</sup>	0.95±0.01 <sup>ba</sup>	0.94±0.00 <sup>aB</sup>	4.38±0.16 <sup>aC</sup>	4.16±0.16 <sup>aBC</sup>	4.16±0.3 <sup>aC</sup>	4.27±0.13 <sup>aB</sup>
10	nd	30.8±0.12 <sup>aA</sup>	nd	31.2±0.06 <sup>aA</sup>	nd	0.93±0.00 <sup>aA</sup>	nd	0.93±0.01 <sup>aA</sup>	-	5.17±0.65 <sup>aC</sup>	-	5.86±0.62 <sup>aC</sup>

Values are mean ±SD (n=5); values sharing same superscript in a column are not statistically significant at  $p < 0.05$ ; <sup>a, b, c, d</sup> indicate significant differences between the different formulations and <sup>A, B</sup> indicate significant differences for the same formulation on the different days of storage. determined by ANOVA and Turkey's test for multiple comparisons. † Abbreviations F1: WF+FF+SF+CO without preservative. F1: WF+FF+SF+CO+Preservative; F2: WF+FF+WB+OO without preservative and F2+P: WF+FF+WB+OO+Preservative. WF: wheat flour. SF: soybean flour. FF: flaxseed flour. WB: wheat bran. OO: olive oil and CO: canola oil. nd: not determined

**Table 3.** Thermal properties of bread made flours mixture and oils during storage: melting heat (apparent) of gelatinized or retrograded starch ( $\Delta H$ ), initial transition temperature ( $T_0$ ), peak transition temperature ( $T_p$ ), final transition temperature ( $T_m$ ).

Storage time (days)	0		3		6	
Formulations	F1	F2	F1	F2	F1	F2
$T_0(^{\circ}\text{C})$	61.64 $\pm$ 0.47 <sup>b</sup>	61.09 $\pm$ 0.00 <sup>b</sup>	42.73 $\pm$ 0.37 <sup>a</sup>	44.26 $\pm$ 0.34 <sup>a</sup>	44.55 $\pm$ 0.95 <sup>a</sup>	43.66 $\pm$ 0.28 <sup>a</sup>
$T_p(^{\circ}\text{C})$	68.81 $\pm$ 0.68 <sup>b</sup>	68.41 $\pm$ 0.99 <sup>b</sup>	55.50 $\pm$ 1.61 <sup>a</sup>	57.72 $\pm$ 0.26 <sup>a</sup>	56.61 $\pm$ 0.67 <sup>a</sup>	55.90 $\pm$ 0.32 <sup>a</sup>
$T_m(^{\circ}\text{C})$	101.12 $\pm$ 2.4 <sup>b</sup>	103.08 $\pm$ 1.06 <sup>b</sup>	71.58 $\pm$ 2.09 <sup>a</sup>	74.67 $\pm$ 0.18 <sup>a</sup>	74.04 $\pm$ 0.26 <sup>a</sup>	73.97 $\pm$ 0.04 <sup>a</sup>
$\Delta T$	39.48 $\pm$ 1.93 <sup>b</sup>	41.99 $\pm$ 1.06 <sup>b</sup>	28.85 $\pm$ 2.46 <sup>a</sup>	30.41 $\pm$ 0.16 <sup>a</sup>	29.49 $\pm$ 1.01 <sup>a</sup>	30.32 $\pm$ 0.23 <sup>a</sup>
$\Delta H$ (J/g solid)	5.94 $\pm$ 0.34 <sup>b</sup>	6.95 $\pm$ 0.55 <sup>b</sup>	1.70 $\pm$ 0.05 <sup>a</sup>	2.11 $\pm$ 0.06 <sup>a</sup>	2.03 $\pm$ 0.29 <sup>a</sup>	2.31 $\pm$ 0.08 <sup>a</sup>
% R	-	-	28.78 $\pm$ 2.54	30.53 $\pm$ 2.25	34.19 $\pm$ 2.87	33.33 $\pm$ 3.75

\* Values are mean  $\pm$ SD (n=5); values sharing same superscript in a row are not statistically significant at  $p < 0.05$ , determined by ANOVA and Turkey's test for multiple comparisons. † Abbreviations F1:

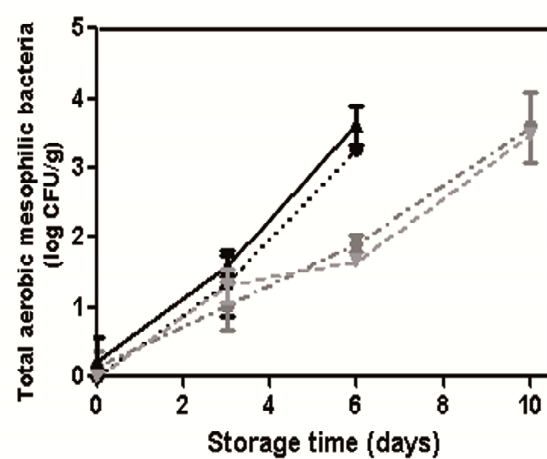
WF+FF+SF+CO; F2: WF+FF+WB+OO. WF: wheat flour, SF: soybean flour, FF: flaxseed flour, WB: wheat bran, OO: olive oil and CO: canola oil.

Table 4. Chemical composition, lipid profile, caloric value and hedonic tests of both wheat flour breads supplemented with different mixtures flours and oils

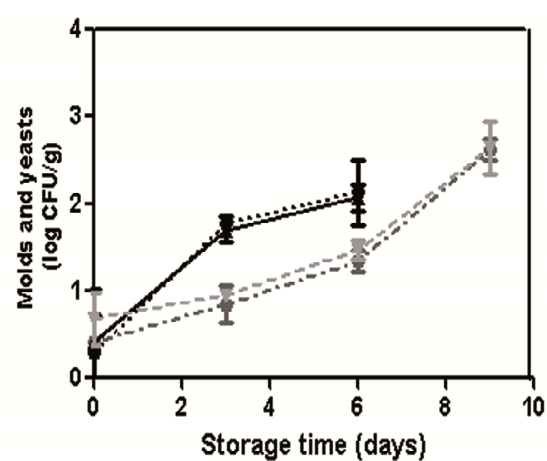
Analysis	Parameters	F1 (WF+FF+SF+CO)	F2 (WF+FF+WB+OO)
<b>Proximal composition</b>	Moisture	32.35±0.07 <sup>a</sup>	33.76±0.43 <sup>b</sup>
	Ash	1.75±0.05 <sup>a</sup>	1.86±0.04 <sup>a</sup>
	Carbohydrates	46.33±0.84 <sup>a</sup>	51.20±0.37 <sup>b</sup>
	Total Fat	2.14±0.24 <sup>a</sup>	2.07±0.30 <sup>a</sup>
	Protein	7.87±0.07 <sup>a</sup>	7.77±0.00 <sup>a</sup>
	Fibre	1.06±0.01 <sup>a</sup>	1.62±0.02 <sup>b</sup>
<b>Fatty acids composition</b>	Palmitic acid C16:0	6.63 ± 0.35 <sup>a</sup>	14.01 ± 0.08 <sup>b</sup>
	Stearic acid C18:0	2.12 ± 0.03 <sup>a</sup>	2.57 ± 0.05 <sup>b</sup>
	Oleic acid C18:1	42.39 ± 0.06 <sup>a</sup>	47.53 ± 0.16 <sup>b</sup>
	Linoleic acid (omega 6) C18:2	24.10 ± 0.85 <sup>b</sup>	22.29 ± 0.02 <sup>a</sup>
	Linolenic acid (omega 3) C18:3	22.69 ± 1.31 <sup>b</sup>	11.22 ± 0.08 <sup>a</sup>
	SFA	10.32 ± 0.05 <sup>a</sup>	17.67 ± 0.04 <sup>b</sup>
	MUFA	42.39 ± 0.06 <sup>a</sup>	48.53 ± 0.06 <sup>b</sup>
	PUFA	47.38 ± 0.02 <sup>b</sup>	33.70 ± 0.02 <sup>a</sup>
	n3	22.69 ± 0.02 <sup>b</sup>	11.22 ± 0.01 <sup>a</sup>
	n6	24.70 ± 0.35 <sup>b</sup>	22.63 ± 0.31 <sup>a</sup>
	n9	42.18 ± 0.06 <sup>a</sup>	47.59 ± 0.05 <sup>b</sup>
	n6/n3	1.09 ± 0.00 <sup>a</sup>	2.01 ± 0.01 <sup>b</sup>
	PUFA/SFA	4.59 ± 0.01 <sup>b</sup>	1.91 ± 0.01 <sup>a</sup>
<b>Sensorial attributes</b>	Colour	7.03±1.32 <sup>b</sup>	6.39±1.69 <sup>a</sup>
	Taste	6.38±2.06 <sup>a</sup>	6.97±1.38 <sup>a</sup>
	Texture	7.42±1.56 <sup>a</sup>	6.67±1.63 <sup>a</sup>
	Overall	6.91±1.59 <sup>a</sup>	6.97±1.33 <sup>a</sup>

Values are mean ±SD (n=3); values sharing same superscript in a row are not statistically significant at  $p < 0.05$ , determined by ANOVA and Turkey's test for multiple comparisons. † Abbreviations WF: wheat flour, SF: soybean flour, FF: flaxseed flour, WB: wheat bran, OO: olive oil and CO: canola oil.

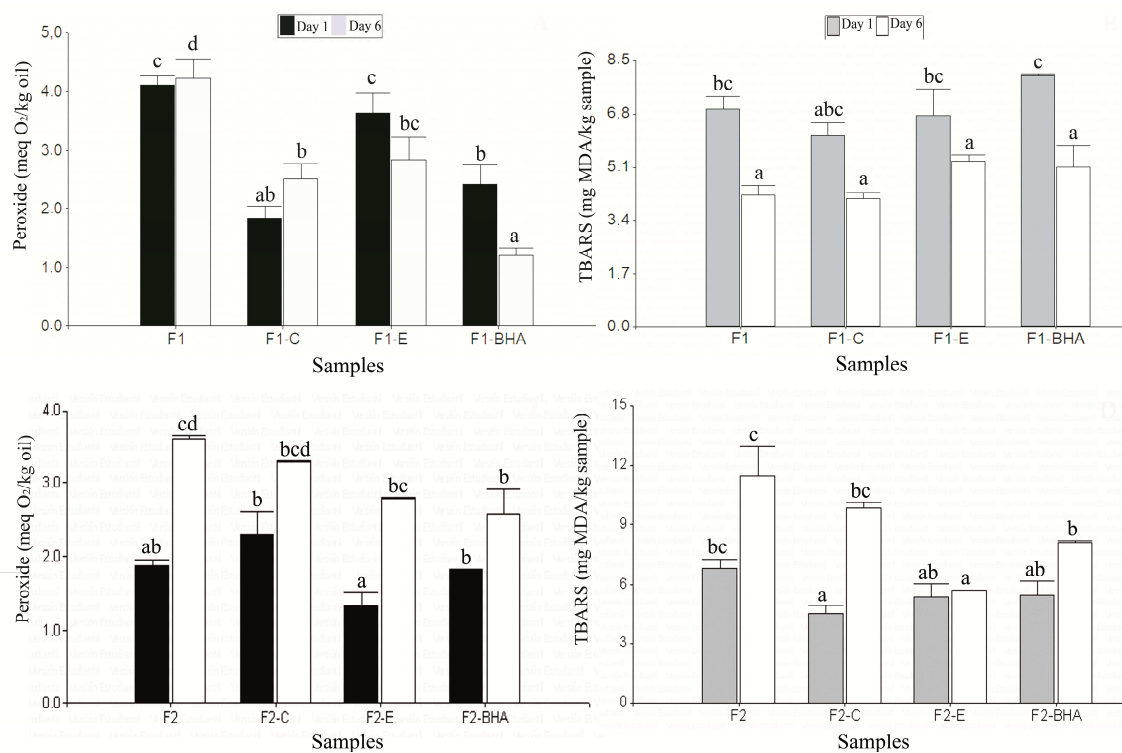
\* Nine-point hedonic scale with 1, 5 and 9 representing extremely dislike, neither like nor dislike, and extremely like, respectively.



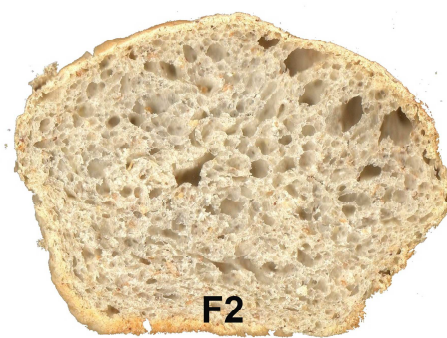
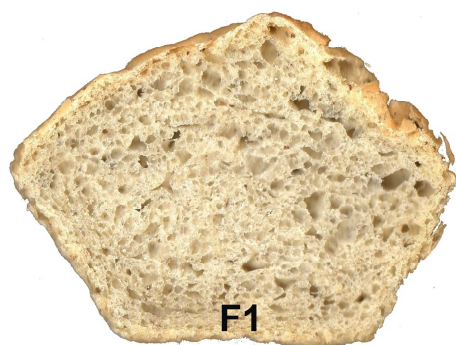
A



B







**Highlights**

- ▶ Ascorbic acid reduces the 40 % of oxidation of multigrain bread with canola oil.
- ▶  $\alpha$ -tocopherol reduces the 50 % of oxidation of multigrain bread made with olive oil.
- ▶ Both formulations presented good sensorial scores.
- ▶ Bread made with flax meal and canola oil duplicated the omega-3 content.
- ▶ The shelf-life of both formulations of multigrain bread was 2 days.